CULTURAL CONDITION FOR GROWTH AND SPORULATION OF *COLLETOTRICHUM FALCATUM* (SUGARCANE RED-ROT FUNGUS)

D.B. OLUFOLAJI

Department of Crop Production, Federal University of Technology,
P.M.B 740, Akure, NIGERIA.

ABSTRACT - Temperature, culture media, nitrogen sources, and inoculation methods were evaluated for their effects on sporulation and growth of the sugarcane red-rot pathogen. When agar media were inoculated with mycelial disks, sporulation was optimum at 15 to 27°C. The optimum range expanded to 30°C ■ cultures on sugarcane stalk extract agar but showed a narrow peak at 27°C when agar media were inoculated directly with spore suspensions. Sugarcane stalk extract agar supported better sporulation than PDA, NA, WA, or any other agar media. Furthermore L-glutamic acid was the best organic nitrogen source for sporulation. Agar cultures incubated at 10°C, reduced the viability of conidia. Longevity of *Colletotrichum falcatum* spores in vitro was better on sugarcane stalk extract agar than on PDA. However, mycelial dry weight growth was better with organic than with inorganic nitrogen sources.

RÉSUMÉ - Les effets de la température, de la composition du milieu de culture, de la source azotée et des méthodes d'ensemencement sur la croissance du champignon agent de la "pourriture rouge" de la canne à sucre ont été déterminés. Quand la culture est inoculée avec un disque mycélien, la production de spores est optimale entre 15 et 27°C. Cette gamme est étendue à 30°C pour les cultures sur extrait de canne à sucre gélosé mais elle devient très étroite autour de 27°C, si l'inoculation est effectuée avec une suspension de spores. La production de spores est meilleure sur un milieu à base de canne à sucre que sur PDA., NA., WA. ou n' importe quel autre milieu solide. En outre, l'acide L-glutamique est la meilleure source d'azote inorganique. L'incubation à 10° C de cultures gélosées diminue la viabilité des conidies. La longévité des spores de *Colletotrichum falcatum* in vitro est plus importante si elles ont été produites sur extrait de canne à sucre gélosé que sur PDA. Toutefois, le poids de mycélium sec obtenu est plus élevé sur sources azotées organiques que sur azote inorganique.

MOTS-CLÉS - *Colletotrichum falcatum, Physallospora tucumanensis*, sugarcane red-rot.

INTRODUCTION

Sugarcane red-rot caused by *Colletrotrichum falcatum* Went is prevalent in the major sugarcane areas of the world, and causes considerable damage to the crop both in cooler and warmer climates (Dickson, 1956).

The fungus exists in two forms; the teleomorph is the Ascomycete, *Physallospora tucumanensis* Speg. and produce ascospores while the anamorph known as *Colletotrichum falcatum* Went. belongs to the Fungi Imperfecti and produces conidia freely (Carvayal & Edgerton, 1944; Dickson, 1956). However, the imperfect stage which produces only conidia is observed in this study. After infection of the leaf midribs, its spores develop mycelium which grows into the leaf tissue, penetrates and secrets toxins which disintegrates the host cells and aid establishment of the fungus. Thereafter, reproduction and production of spores take place. Ascospores are only produced under special and limited conditions while conidia are produced most of the time and under any condition, (Cavayal & Edgerton, 1944; Frohlich & Rodewald 1970).

Red-rot disease causes dry and wilted leaves, reduces sugarcane stands in a plantation and also reduces the sugarcane stands availability in the sugarcane due to sucrose inversion (Dickson, 1956; Frohlich & Rodewald, 1970).

Several studies on the disease have been focused on controlled measures such as selection and use of resistant varieties (Sattar et. al., 1982, Steib & Chilton, 1951). Informations on cultural conditions which promote growth and sporulation which could be a prerequisite for future studies have not been reported in any recent work on *C. falcatum*.

The present paper reports on an investigation on the optimum temperature, appropriate media, method of cultivation and nitrogen sources for growth and sporulation of the sugarcane red-rot pathogen.

MATERIALS AND METHODS

The isolates used in this study were obtained from a diseased sugarcane stem from the experimental site of the Nigerian Sugar Company Estate, Bacita, Nigeria. Its pathogenicity was confirmed by tests on healthy sugarcane plants in the greenhouse and laboratory. The fungal stock culture were maintained on potato dextrose agar (PDA). The solid media used for the investigations were PDA, water agar (WA), sugarcane stalk extract agar (SSEA), commeal agar (CMA), malt extract agar (MEA), nutrient agar (NA), yeast extract agar (YEA), dextrose agar (DA) and potato agar (PA). Media were prepared according to manufacturers guidelines, except SSEA which contained 10g of sugarcane pulp and 20g of agar in 1 litre of solution. The media were sterilized by autoclaving at 1.1kg/cm^2 (121°C) for 15 min. and 15ml were dispensed into 9cm Petri dishes.

The basal liquid medium for the nitrogen nutrition study was the one used by Kurtz & Fergus (1964) and Oritsejafor (1986) and consists of $MgSO_4.7H_2O$, 0.5g; KH_2PO_4, 1.0g; thiamin, 0.1mg; biotin, 0.005mg; Fe, 0.2mg; Zn, 0.2mg; and Mn, 0.2mg. in a quantity to yield an amount of nitrogen equal to that of 2g of asparagine per litre. For organic nitrogen media the carbon supplement sources of the medium were added to provide a total of 4g of carbon per litre (glucose carbon plus the amount of carbon added in the organic nitrogen compound).

All components of the medium except the nitrogen sources were combined at twice the desired final concentration, dispensed in 25ml parts in 250ml Erlenmeyer

flasks and sterilized by autoclaving. 25 ml of the nitrogen solution at twice the desired final concentration were sterilized separately in test tubes, cooled and asceptically added to the cooled medium. Urea solutions were sterilized by seive filtration. All solutions were adjusted to the required pH by added sterile 0.1N HCl or 0.1N NaOH.

Media were inoculated either with mycelial disks or with spore suspension. Mycelial disk inoculations were made by using 6-mm diameter disks cut with a sterile No. 34 cork borer from 4-day old cultures of the fungus on PDA. For experiments involving solid media the disks were placed at the centre of the agar plates, otherwise with liquid culture, disks were dropped into the flasks.

With spore suspension inoculations, 0.1ml of spore suspension (5,000 conidia/ml) was spread over the surface of the Petri dish agar medium. All cultures were incubated for 7 days at each 3° intervals from 3 to 33°C. In the nitrogen studies, shaked and stationary liquid cultures were compared for growth and sporulation at 27°C. The shaked cultures were placed in a shaker incubator at 27°C at 80 rpm. Treatments were replicated four times and nitrogen free control media were included.

Conidia were collected from the solid media by pouring 10ml of distilled water over the fungal colony, allowing it to stand for about 1 min. Spores were dislodged with sterile camel's-hair brush and the water suspension was shaken and sieved through double layers of cheese cloth to remove agar and mycelia debris. A haematocytometer was used to count the conidia. Mycelia from liquid cultures was carefully removed from the flasks and placed in clean Petri dishes containing 10ml of distilled water. The mycelium was rinsed three times with distilled water and collected on a previously dried filter paper and dried overnight at 80°C and weighed. The pH of the filtrates was determined with a Beckman pH meter.

The longevity of *Colletotrichum* conidia was determined by placing sporulating cultures maintained on PDA and SSEA in storage at 10°C. Each month for 12 months, samples of conidia were removed, washed, and germinated in water on microscope slides at 27°C and about 100% RH.

Sporulation was also studied on sugarcane pulp. A spore suspension 10,000 conidia/ml was spread on the pulp surface and incubated at 27°C and 100% RH, for one week. Then the surface was washed under running tap water and brushed with a sterile camels' hair brush to remove conidia and leave only the mycelium embedded within the pulp tissues. The pulp was incubated for another week under the same conditions, and then examined for the presence and amount of sporulation.

Data analysis:

Duncan's Multiple Range Test (Little & Hill, 1975). This test is most widely used when several multiple range tests are available. It gives protection against making mistakes inherent in the indiscriminate use of the L.S.D. tests.

Here it is used for making all possible comparison among the fungus yields under various nitrogen sources. The test however involves the calculation of **shortest significant differences** (SSD) for all possible relative positions between treatment mean in ascending arrays, using the formular below, and starting from analysis of variance under completely randomised design.

a) $LSD_{.05}=t\sqrt{\frac{2S^2}{r}}$ t = obtained from studentised factor table.

b) Calculation of SSD for relative position in the array of means. Using significant studentised factor from studentised table. Value of R, is read at 5% level of significant from the table and the value is used for the equation:-

SSD = R(LSD). *SSD = Duncan Multiple Range Values which could be used to separate the treatment means.

RESULTS

Effects of temperature on sporulation

Temperatures from 15 to 27°C supported the production of large number of conidia when mycelial disks were plated on PDA (fig. 1). There was no significant difference in sporulation within these temperatures but sporulation was significantly lower below 12 or above 30°C. There was no growth or sporulation at 30°C but a slight one was obtained at 6°C. This pattern of temperature effects was similar on all the tested media. When cultures were inoculated with conidia, sporulation increased greatly from 15 to 27°C, and decreased from 27 to 33°C (Fig. 2). Sporulation was not observed

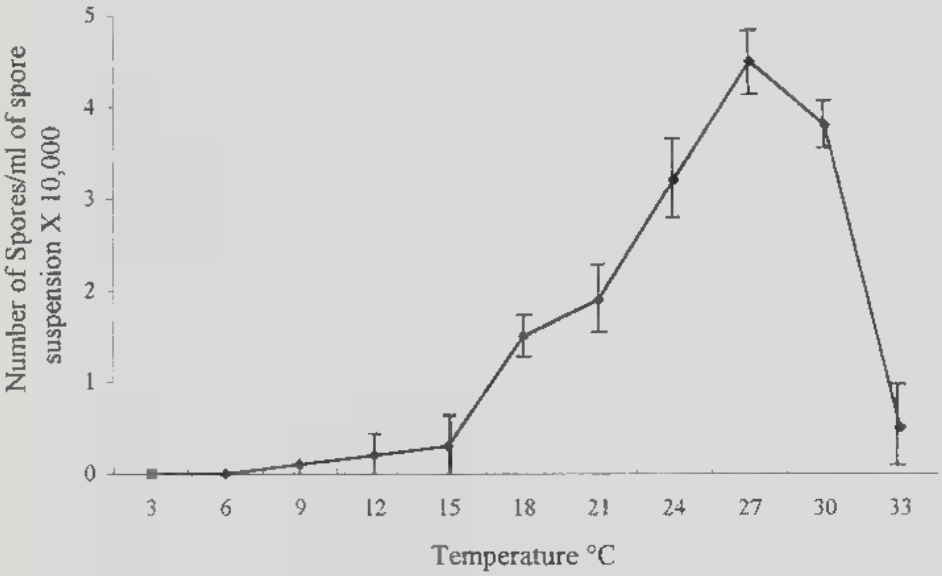

Fig. 1: Sporulation of cultures of *Colletotrichum falcatum* grown from mycelial agar disks, placed on sugarcane stalk extract agar and incubated at various temperatures for 7 days.

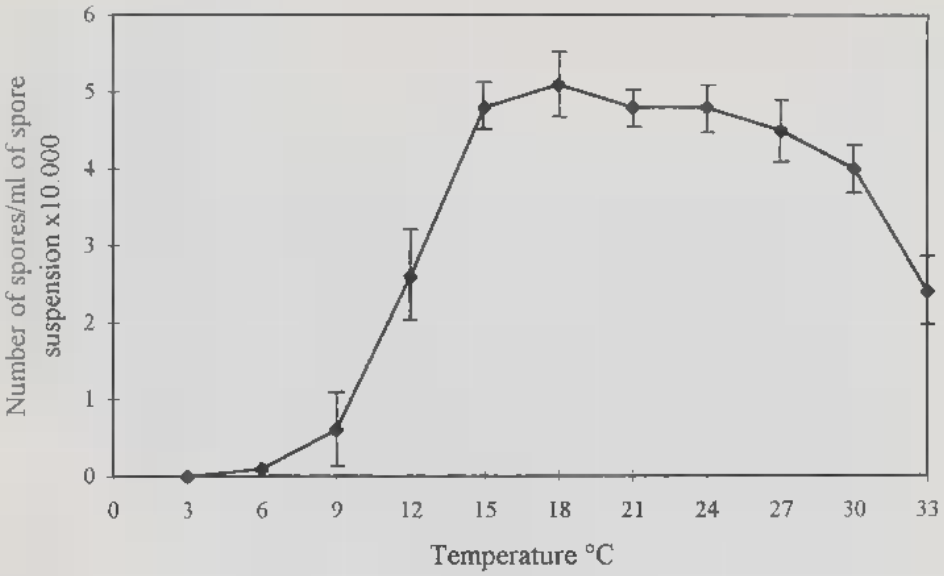

Fig. 2: Sporulation of cultures of *Colletotrichum falcatum* grown from spores sown on sugarcane stalk extract agar and incubated ■ different temperatures for 7 days.

from 3 to 9°C, while the greatest amount of conidia was produced at 27°C at about 43,000 conidia per millilitre of distilled water. The amount of conidia produced at this temperature was significantly greater than for other temperatures.

It was also observed that a large amount of sporulation occurred at 9, 12 and 33°C.

Effect of medium on sporulation

When agar cultures were initiated with mycelial disks and incubated at 18°C for 7 days, *C. falcatum* produced the greatest amount of spores on SSEA (Fig. 3). Spore production on SSEA was about twice that on NA, YEA, CDA, CMA and PA. The proportions produced on PDA, MEA and WA as compared with SSEA were approximately 0.75, 0.75 and 0.25 respectively. Furthermore, the mycelium was denser on SSEA than on any other medium.

On the agar media inoculated with spores, and incubated at 27°C the sporulation pattern differed from that on media inoculated with mycelial disks. SSEA medium surpassed all others in promoting sporulation but there was lesser sporulation generally than on media inoculated with mycelium (Fig. 3 and 4). Sporulation on SSEA was twice as much as that on NA, YEA, CMA and PA. Sporulation on PDA, MEA, CDA and WA as compared to SSEA was 0.80, 0.75, 0.20 and 0.15 respectively (Fig. 4).

On nearly all the media inoculated with mycelial cultures were able to produce viable spores earlier and in larger quantity than spores inoculated on (Fig. 5). L-glutamic acid supported significantly greater sporulation than other sources (Table 1). This was followed closely by DL-aspartic acid for the first 30 days of growth. Glycine,

	10 days			20 days			30 days		
	Mycelium mg/L	No. of Spores/ mg of mycelium	pH	Mycelium mg/L	No. of Spores/ mg. of mycelium	pH	Mycelium mg /L	No. of Spores/ mg. of mycelium	pH
L-Arginine	161d	70b	5.4	181d	75bc	6.7	194c	100c	6.1
DL-Alanine	200b	69b	6.3	210cb	77b	6.2	198c	98c	5.8
Glycine	210b	71b	5.1	231b	82b	6.4	221b	110c	6.4
L-Glutamic acid	182c	80a	5.1	210c	110a	6.8	194c	140a	6.1
DL-Isoleucine	110f	56cd	3.6	121f	77b	7.2	96g	110c	7.4
L-Methionine	200b	72b	4.2	236b	78b	6.9	221b	98c	7.3
Asparagine	281a	68b	4.6	360a	79b	7.4	380a	108c	8.2
DL-Aspartic acid	103g	70b	5.2	181d	84b	6.8	171cd	121b	7.9
L-Leucine	98h	61c	6.1	102g	78b	7.7	126ef	103c	8.2
DL-Threonine	116fg	66c	4.4	131f	70c	6.8	141e	89d	8.1
DL-Histidine	136ef	42e	5.3	162e	56d	5.4	116f	78e	8.4
Urea	90h	50d	6.1	111g	59d	6.6	86g	68ef	6.8
L-Tryptophane	161d	56cd	5.8	194ec	62d	6.3	214b	81de	7.1
$(NH_4)_2SO_4$	62i	55cd	4.8	81h	64d	4.5	66h	94cd	6.6
$NaNO_3$	77hi	40e	5.2	80h	52de	6.2	72g	77e	6.0
NH_4Cl	71hi	36e	6.3	94h	48e	6.1	110f	52f	6.1
$NaNO_3$	78hi	41e	4.4	140f	48e	5.6	120f	69ef	6.1
Control	42j	28f	6.6	56 l	39f	6.1	66h	54f	5.2

Table 1:- Dry Weight of mycelium and spore production by *Colletotrichum falcatum* and the pH of the liquid culture media of different nitrogen sources. Y = Mean values (in the same column) not followed by the same letter are significantly different (P = 0.05) according to Duncan's multiple range test. Initial pH was 7.0.

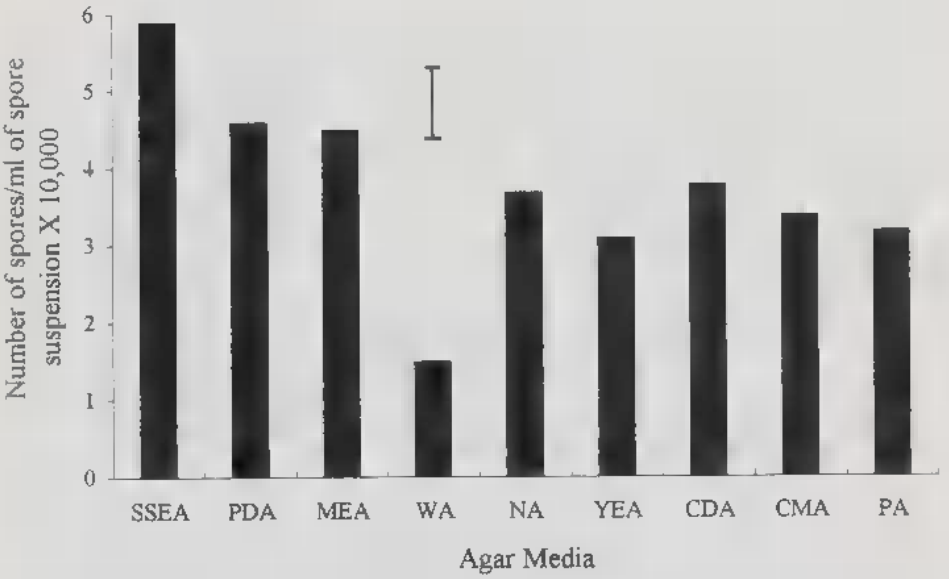

Fig. 3: Sporulation of cultures of *Colletotrichum falcatum* when mycelial disks were plated on different agar media and incubated ■ 10°C for 7 days. I = Least significant difference (P = 0.05).

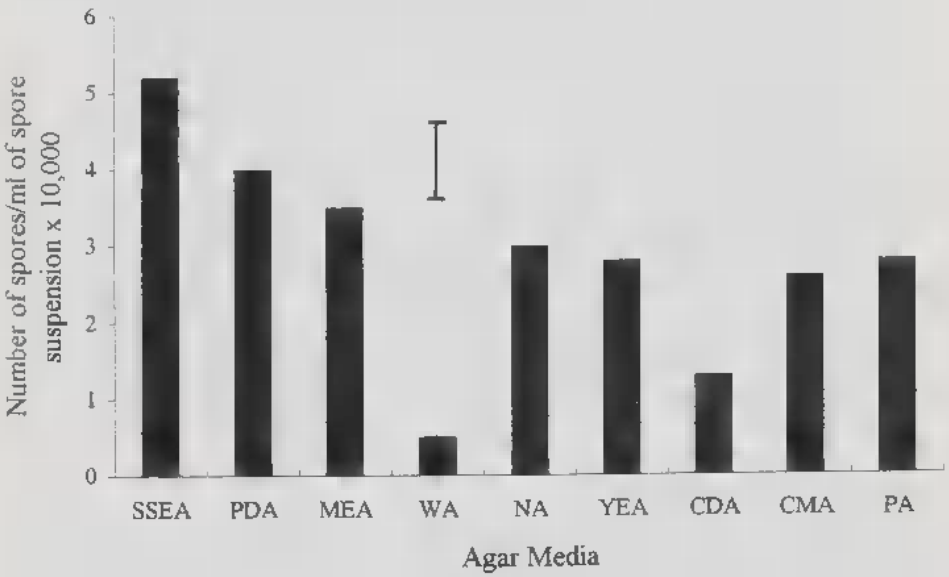

Fig. 4: Sporulation of cultures of *Colletotrichum falcatum* when spores were sown on different agar media and incubated at 27°C for 7 days. I = Least significant difference (P = 0.05).

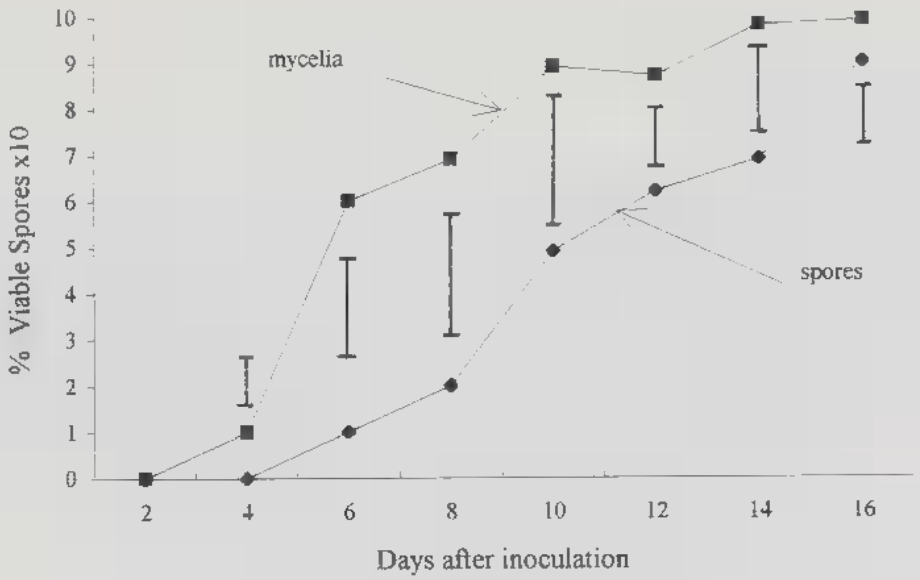

Fig. 5: Viable spores produced by *Colletotrichum falcatum* in cultures formed by spores and mycelial seeding methods I = Least significant difference (P = 0.05)

asparagine and DL-isoleucine also supported a considerable amount of sporulation (Table 1). The organic nitrogen group was significantly better than the inorganic group for sporulation.

Effect of culture age ▬ spore viability

When sporulating cultures were maintained on PDA test tube slants at 10°C there wan no significant decrease in viability during the first 3 months of storage (Fig. 6).

Months 4 to 6 were not significantly different from each other but showed decreased viability, when compared to the first 3 months. There was a further decrease in viability from the 7th to the 10th month, and no conidia viable at the 11th or 12th month.

On the other hand, when cultures were stored on SSEA test tube slants the percentage of viable conidia decreased significantly only from the 4th to the 9th month, with slightly more decrease till 12 months (Fig. 7). Generally viability was greater on SSEA than PDA cultures.

Growth on various nitrogen sources

The shaked and stationary cultures did not give significant different results. Thus, means of the two treatments were combined for the presentation in Table 1. Growth of *C. falcatum* was better in the organic than inorganic nitrogen sources, with asparagine significantly leading all other sources. In all the inorganic nitrogen group

Fig. 6: Germination of spores harvested ■ monthly intervals from cultures of *Colletotrichum falcatum* stored on potato dextrose agar at 10°C. I = Least significant difference (P = 0.05).

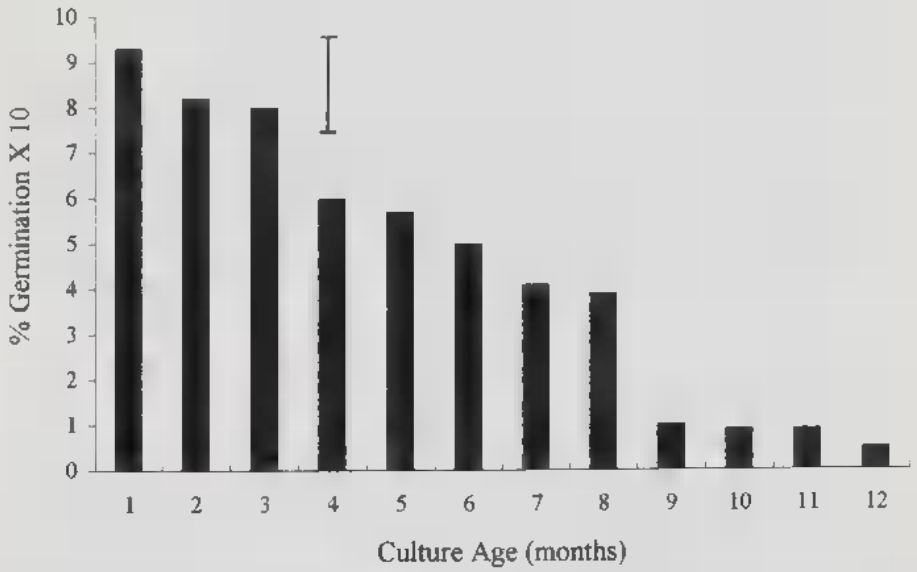

Fig. 7: Germination of spores harvested at monthly intervals from cultures of *Colletotrichum falcatum* stored on sugarcane stalk extract agar at 10°C. I = Least significant difference (P = 0.05).

($NaNO_2$) and NH_4SO_4) supported the least growth. Autolysis and loss in mycelial dry weight started earlier with $NaNo_3$ (after 15 days) than in asparagine or $NaNO_2$ (after 25 days) (Fig. 8). Throughout the experiments, cultures on asparagine had the greatest amount of growth (Fig. 8).

Nitrogen sources influenced the final pH of the medium. The pH of the asparagine medium rose from 4.6 to 8.2 at the end of the experiment while that of $NaNo_3$ rose from 5.6 to 6.5. The control flask had scanty growth and it's pH decreased from 6.6 to 5.2.

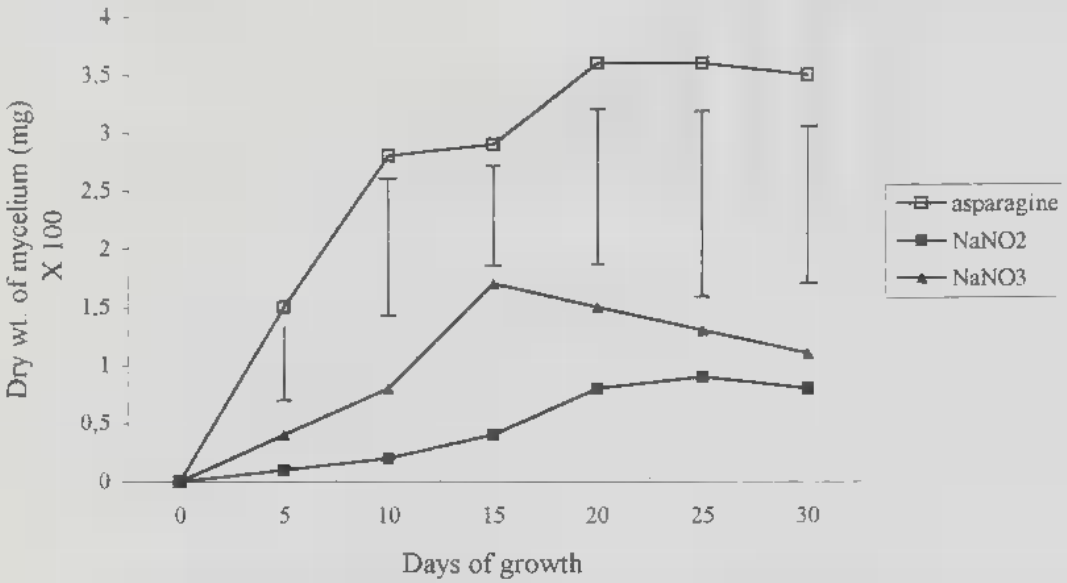

Fig. 8: Mycelium produced by *Colletotrichum falcatum* in liquid culture containing asparagine, $NaNO_3$ and $NaNO_2$, as sources of nitrogen. I = Least significant difference (P = 0.05).

DISCUSSION

As reported on some other fungi, production of spores of *C. falcatum* could be carried out on media inoculated with either mycelial or conidia. Olufolaji (1984) demonstrated this with *Curvularia pallescens* Boed. There are crossed influences of temperature on method of inoculation. Higher temperatures tolerance was noted in cultures started from conidia than in those started from mycelium. This seems to agree with the findings of Olufolaji (1984) for *C. palescens*. There is the possibility that conidia need higher temperature than mycelium for initiating germination and growth, before the sporulation can take place. In the already available mycelium, elongation would only be needed and this requires lesser temperature and energy (Singh & Wood, 1956; Chi & Hanson, 1964; Olufolaji 1984; and Oritsejafor, 1986).

There was also an interaction of medium and inoculation method upon sporulation. Nutrient rich media support sporulation differently in different methods of

initiating cultures. However SSEA seemed to favour sporulation by either method of inoculation. Olufolaji (1984) and Chi & Hanson (1964) had opposing results with *C. pallescens* and *Fusarium* spp respectively. Chi & Hanson (1964) and Oritsejafor (1986), observed that sporulation of *Fusarium* cultures initiated with conidia needed more nutrients than those initiated with mycelial disc. Also, in *C. falcatum* nutrient rich media were needed for sporulation more in one inoculation method than the other. It thus became evident that mycelial initiated cultures sporulated earlier than conidia initiated ones, this is because in mycelium, it only requires production of conidiophore before conidia production, while conidia will need a lot of nutrient and energy for germination to hyphae, to mycelium, before conidiophore prior sporulation.

The finding that L-glutamic acid was a superior organic source of nitrogen, and that among inorganic sources $NaNO_3$ was best, agrees with those reported for several imperfect fungi (Olufolaji, 1984;. Tandon & Chandra, 1990). Sporulation on sugarcane pulp was equivalent to conidia initiation through the mycelium planting method, since the washed pulp contained initially mycelium but no conidia in its tissues. In agreement with this, the temperature conditions that favoured sporulation by the mycelium planting method also promoted that by washed sugarcane pulp. Conidia regularly lost their viability during ageing in culture, as it had been shown for *C. pallescens* (Olufolaji, 1984). However, unlike *C. pallescens*, some of the conidia of *C. falcatum* were still viable after 12 months of storage at 10°C. This could be due to more food reserve in the conidia (Carvayal & Edgerton, 1944) in contrast to those of *C. pallescens* and *Fusarium* spp. (Olufolaji, 1984; Chi & Hanson, 1964). It was evident that SSEA medium promoted the lowest maintenance of spore of *C. falcatum* culture.

Collectotrichum falcatum was able to grow very well on many organic and inorganic nitrogen sources. This may afford it a capability of surviving well on a wide variety of substrates as it is the case for many fungi (Chi & Hanson 1964; Olufolaji, 1984; Oritsejafor, 1986; Pfender & Wootka, 1987). The optimum nitrogen source for *C. falcatum* growth is asparagine but this is usually not the one preferred by most parasitic fungi, otherwise L-leuchine and Urea, which are poor sources of nitrogen for most fungi, also gave poor growth in this study. However, $NaNO_3$ which supported greatest growth in the inorganic nitrogen sources is among the best sources for most fungi (Cochrane 1958; Kurtz & Fergus 1964; Olufolaji 1984; Oritsejafor, 1986, Tardon & Chandra, 1990).

REFERENCES

CARVAYAL E., and EDGERTON C.W., 1944 - The perfect stage of *Colletotrichum falcatum*. *Phytopathology* 34: 206-213.

CHI C. C. and HANSON E. W., 1964 - Relation of temperature pH and nutrition to growth and sporulation of *Fusarium* spp from clover. *Phytopathology* 54: 1053-1058.

COCHRANE V. W., 1958 - Physiology of Fungi John Wiley and Sons. New York.

DICKSON J. G., 1956. - Diseases of Field Crops: McGraw Hill Co. London. Pp. 216-218.

FROHLICH G. and RODEWALD W., 1970. - *Pests and diseases of Tropical Crops and their control*. Pegamon Press Oxford. Pp. 233-234.

HURTZ E.B. and FERGUS C.L., 1964 - Nitrogen nutrition of *Colletotrichum cocoodes*. *Phytopathology* 54: 691-693.

LITTLE T. M. and HILLS J.F., 1975 - Statistical methods in agricultural research. University of California, Davis U.S.A. Pp. 50-51.

OLUFOLAJI D.B., 1984 - Sporulation and growth of *Culvularia pallesccens* as affected by media temperature and nitrogen sources. *Phytopathology* 74: 260-263.

ORITSEJAFOR J.J., 1986 - Carbon and nitrogen nutrition in relation to growth and sporulation of *Fusarium oxysporum*. *Trans. Brit. Mycol. Soc.* 87 (4): 519-624.

PFENDER W.F. and WOOTKA S.L., 1987 - Production of Psendothecia and ascospores by *Pyrenophora triticirepentis* in response to macro-nutrients concentration. *Phytopathology* 77: 1213-1216.

SATTAR M.A., RAMAKISHNA RAO P. and NAGESWARA RAO P., 1982 - Screening sugarcane clones for resistance to red-rot disease. *Sugarcane Path. Newsletter* 28: 11-14.

SINGH R.K. and WOOD R.S.K., 1956 - Studies in the physiology of parasitism. The production and properties of pectic enzymes secreted by *Fusarium moniliforme* sheldon *Ann. Bot.* 20: 89-103.

STEIB R.J. and CHILTON S.J.P., 1951 - Infection of sugarcane stalks by the red-rot fungus *Physallospora tucumanensis* Speg. *Phytopathology* 41: 522-528.

TANDON R.N. and CHANDRA R.P., 1990 - Nutritional factors affecting imperfect fungi. *National Acad. of Sci. India* 61 (B): 391-398.